A reversible protein phosphorylation system is present at the surface of infective larvae of the parasitic nematode *Trichinella spiralis*

Vincent P. Smith[1], Murray E. Selkirk, Kleoniki Gounaris*

Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY, UK

Received 8 August 2000; revised 20 September 2000; accepted 21 September 2000

Edited by Matti Saraste

Abstract ***Trichinella spiralis*** **infective larvae have externally oriented enzymes catalysing reversible protein phosphorylation on their surface. Incubation of larvae with exogenous ATP resulted in phosphorylation of surface bound and released proteins. Exposure of the parasites to bile, a treatment which renders them infective for intestinal epithelia, resulted in increased release of protein and an altered profile of phosphorylation. Both serine/threonine and tyrosine phosphorylation and dephosphorylation reactions took place at the parasite surface. Examination of the structural characteristics of the larvae following exposure to bile showed that the non-bilayer surface coat was not shed but was structurally reorganised.**

Key words: Ecto-enzyme; Protein phosphorylation; *Trichinella spiralis*

1. Introduction

The nematode parasite *Trichinella spiralis* can infect a wide variety of mammalian hosts. Infection is initiated when parasites invade the mucosa of the small intestine, where they reside within rows of columnar epithelial cells [1]. The mechanism of cellular invasion is undetermined, although infectivity is dependent upon activation of parasites by exposure to intestinal contents or bile [2]. Although the parasites exhibit a broad host range, invasion by this stage appears to be restricted to epithelial cells [2]. This selectivity may depend upon the capacity for signal transduction processes and the requirement of specific interactions between parasites and host cells.

Recently, we demonstrated that *T. spiralis* larvae secrete at least two distinct serine/threonine protein kinases [3]. Protein kinases could have several roles in invasion and migration of larvae within the mucosal epithelium. Activation of signal transduction pathways is known to be essential for internalisation of pathogens in host cells, and is invariably associated with phosphorylation of cellular proteins, notably cytoskeletal elements and components of signalling pathways such as the mitogen-activated protein kinase cascade [4]. The vast majority of this work has focused on bacteria, although there is a growing literature on parasitic protozoa. Less is known about externally oriented surface enzymes of pathogens, although the potential involvement of such ecto-protein kinases and/or phosphatases in signal reception and transduction as well as parasite–host cell interactions in general could be of considerable significance.

We report here that infective larvae of *T. spiralis* possess ecto-protein kinase and phosphatase activities catalysing the phosphorylation/dephosphorylation of secreted and membrane bound parasite proteins. The enzymes are sensitive to inhibitors of both serine/threonine and tyrosine kinases and phosphatases. Activation of infectivity of the larvae resulted in changes in the activity and substrate specificity of these enzymes. It therefore appears that this intracellular parasite has a complete and active reversible protein phosphorylation system on its surface, suggestive of an involvement with host cell signal transduction pathways.

2. Materials and methods

2.1. Parasites

Infective larvae of *T. spiralis* were recovered from outbred rats 2 months after oral infection as previously described [3]. In some experiments, separate groups of parasites were immediately incubated in either 5% bovine bile (Sigma) in phosphate-buffered saline (PBS) (pH 7.4) or PBS alone for 1 h at 37°C. Bile was removed prior to analysis by exhaustively washing the worms in PBS.

2.2. Assay for phosphorylation of surface bound and secreted proteins

In a standard assay, groups of 10 000 parasites were rinsed three times with 25 mM Tricine pH 8.0, before resuspension in 90 ml of this buffer containing 10 mM $MgCl_2$ and 10 mM 1,4-dithiothreitol (DTT) (phosphorylation buffer). The parasites were then preincubated for 30 min at 37°C before the addition of 20 μCi of [γ-^{32}P]ATP (ICN) diluted to a volume of 10 μl with phosphorylation buffer. After incubation for 30 min at 37°C with gentle agitation, secreted proteins were recovered by collecting the phosphorylation buffer, rinsing the worms twice in 200 μl PBS and pooling the liquid samples. The parasites were washed twice further in PBS and their membrane bound proteins solubilised by incubation in 0.5 ml PBS pH 7.4, 0.5% cetyltrimethylammonium bromide (CTAB) for 1 h at room temperature. All proteins were precipitated in 10% TCA, washed with cold acetone, resuspended in sample buffer and analysed by 12.5% SDS–PAGE under reducing conditions. Labelled phosphoproteins were visualised by autoradiography.

The effect of protein kinase and phosphatase inhibitors on phosphorylation was assessed by the inclusion of these compounds in the buffer in which the worms were preincubated prior to the addition of ATP. Genistein (4′,5,7-trihydroxyisoflavone), A-3 hydrochloride (*N*-(2-aminoethyl)-5-chloronaphthalene-1-sulphonamide HCl) and staurosporine (*Streptomyces* sp.) were all purchased from Calbiochem. Okadaic acid (*Procentrum concavum* sp.) and sodium orthovanadate were from Sigma. The final concentrations of the inhibitors were as follows: genistein at 50 μM, A-3 at 50 μM, staurosporine at 10 μM,

*Corresponding author: Fax: (44)-20-7594 5207.
E-mail: k.gounaris@ic.ac.uk

[1] Present address: Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK.

Abbreviations: CTAB, cetyltrimethylammonium bromide; $^{32}P_i$, radiolabelled inorganic phosphate; A-3, *N*-(2-aminoethyl)-5-chloronaphthalene-1-sulphonamide HCl; DTT, 1,4-dithiothreitol

0014-5793/00/$20.00
PII: S0014-5793(00)02094-9

okadaic acid at 2 μM and sodium orthovanadate at 1 mM. Inorganic ^{32}P and [α-^{32}P]ATP were used instead of [γ-^{32}P]ATP as indicated. All radionuclides were purchased from ICN. Hexokinase and apyrase were purchased from Sigma and used at concentrations of 50 U/ml and 100 U/ml, respectively.

2.3. Assay for release of secreted proteins

Groups of 10 000 parasites preincubated in bile or PBS as described above were resuspended in 100 μl 25 mM Tricine pH 8.0, 10 mM $MgCl_2$, in the presence or absence of 10 μM ATP. They were incubated at 37°C for 2 h and at several time points, pairs of 5 μl aliquots of buffer were removed from each tube and assayed for protein content using the BCA microplate assay (Pierce).

2.4. Electron microscopy

Freeze fracture and thin section electron microscopy was performed as previously described [5]. Replicas and thin sections were examined using a Philips EM 301G transmission electron microscope at 100 kV.

3. Results

Incubation of live infective larvae with [γ-^{32}P]ATP resulted in the phosphorylation of a significant number of secreted parasite proteins. The most prominent phosphoproteins resolved as doublets at 110–115 kDa, 70–80 kDa and 47–60 kDa, with additional species at 40, 36 and 29 kDa, and minor phosphoproteins of lower molecular mass (Fig. 1, lane 1). Incubation in the presence of genistein resulted in decreased incorporation of ^{32}P into most of the phosphoproteins, indicative of tyrosine phosphorylation (lane 2). Inclusion of sodium orthovanadate in the incubation medium gave rise to a profound increase in protein phosphorylation (lane 3). A differential effect was observed when serine/threonine kinase inhibitors were employed. Both staurosporine (lane 4) and the synthetic kinase inhibitor A-3 (lane 5) had similar effects. Thus, phosphorylation of the proteins at 40 and 36 kDa was not affected, whereas labelling of proteins in the 47–60 kDa range was diminished, and phosphorylation of all other proteins severely inhibited. Addition of okadaic acid (lane 6) at a concentration sufficient to inhibit both type 1 and 2 serine/threonine phosphatases resulted in an increase of phosphate incorporation into the proteins in the 70–80 kDa range, and two additional phosphoproteins at 47 and 60 kDa were now clearly discernible. It is worth noting that phosphorylation of the 70–80 kDa doublet was enhanced by both sodium orthovanadate and okadaic acid, suggestive of phosphorylation on both tyrosine and serine/threonine residues. Furthermore, addition of okadaic acid inhibited phosphorylation of several proteins, notably those at 40 and 47–60 kDa, suggesting a potential regulatory role for serine/threonine phosphatases on protein kinase activity.

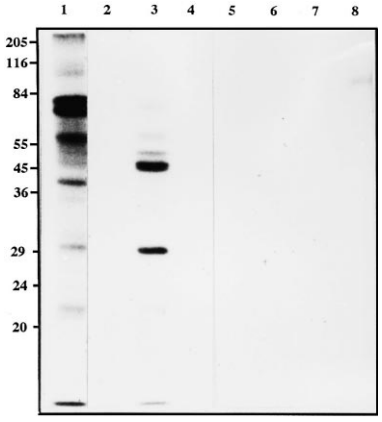

Fig. 2. Phosphorylation of released proteins is due to ecto-enzymes. Lane 1: control reaction; other reactions were performed as follows: lane 2: in the absence of $MgCl_2$ and DTT; lane 3: in the absence of DTT; lane 4: in the absence of $MgCl_2$; lane 5: phosphorylation reaction carried out with [α-^{32}P]ATP; lane 5: phosphorylation reaction carried out with $^{32}P_i$; lane 6: in the presence of 100 U/ml apyrase; lane 7; in the presence of 50 U/ml hexokinase.

Phosphorylation of extrinsic proteins was completely dependent upon magnesium (Fig. 2, lanes 2 and 4). Interestingly, in the absence of DTT, prominent phosphorylation of proteins at 47 and 29 kDa alone was observed, with minor labelling of a few other proteins (lane 3). In order to ascertain that the enzymes involved were indeed present at the surface of the parasite with their catalytic sites externally oriented, we carried out experiments in which [γ-^{32}P]ATP was replaced by [α-^{32}P]ATP or inorganic ^{32}P. Under these conditions, there was complete absence of phosphate incorporation into extrinsic proteins (lanes 5 and 6). Furthermore, inclusion of the ATP-utilising enzymes apyrase (lane 7) or hexokinase (lane

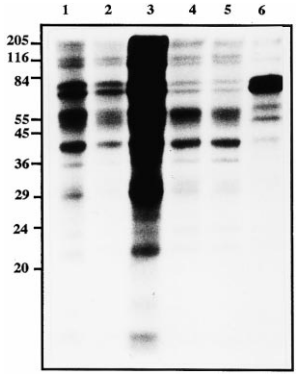

Fig. 1. Phosphorylation of proteins released by *T. spiralis*. Lane 1: control reaction (no inhibitor). Lanes 2–6 show the effects of 50 μM genistein, 1 mM orthovanadate, 10 μM staurosporine, 50 μM A-3 and 2 μM okadaic acid, respectively. Proteins were resolved by SDS–PAGE under reducing conditions. The M_r of marker proteins is shown in kDa.

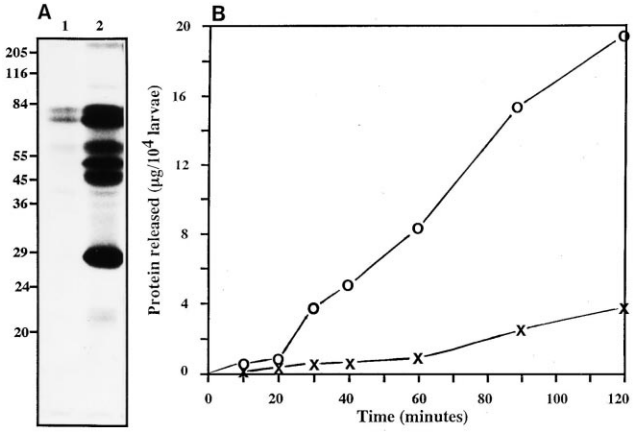

Fig. 3. Effect of bile treatment on phosphorylation of released proteins. (A) Untreated (lane 1) and bile-treated (lane 2) samples. (B) Quantitative analysis of protein secreted by untreated (x) and bile-treated (o) larvae as a function of time.

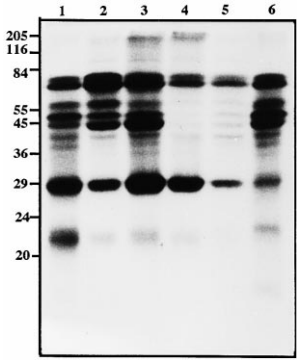

Fig. 4. Effect of inhibitors on phosphorylation of proteins released by bile-activated parasites. Lanes as in Fig. 1.

8) inhibited protein phosphorylation, again confirming that this is an extrinsic process.

Exposure of *T. spiralis* larvae to bile has been shown to be necessary for activation and acquisition of infectivity [2], and we therefore investigated whether phosphorylation of the secreted parasite proteins was affected by this treatment. Secreted proteins from equal numbers of untreated (Fig. 3A, lane 1) or treated larvae (Fig. 3A, lane 2) were collected following phosphorylation and examined by SDS–PAGE and autoradiography. There was a massive increase in phosphorylation of secreted proteins following bile treatment when compared to the control samples, and it was also evident that exposure to bile altered the profile of protein phosphorylation (Fig. 3A; also compare Fig. 1, lane 1 and Fig. 4, lane 1). In addition, exposure to bile resulted in a significant increase in the amount of total protein secreted by larvae (Fig. 3B).

Further experiments highlighted differential effects on phosphorylation of proteins released by the parasites following bile treatment (Fig. 4). Genistein appeared to have an inhibitory effect on phosphorylation of proteins at 40, 38, 29 and 20 kDa, and interestingly sodium orthovanadate also appeared to inhibit phosphorylation of the latter protein. Perhaps the most striking effect was observed with serine/threonine kinase inhibitors. Both staurosporine and A-3 (lanes 4 and 5, respectively) inhibited phosphorylation of the 60, 50, 43, 40, 38 and 22 kDa proteins, whereas A-3 alone inhibited phosphorylation of the 29 kDa protein. Addition of okadaic acid (lane 6) resulted in enhanced phosphorylation of all proteins except those at 29 and 22 kDa.

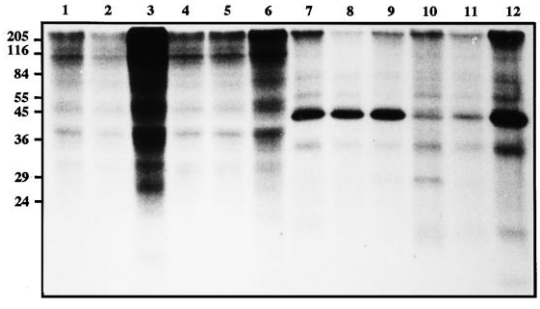

Fig. 5. Phosphorylation of surface membrane bound proteins by ecto-enzymes. Untreated (lanes 1–6) or bile-treated (lanes 7–12) parasites. Lanes 1 and 7: control reactions (no inhibitors). Lanes 2–6 and 8–12: inhibitors assessed as in Figs. 1 and 4.

To investigate whether any surface membrane bound proteins were also phosphorylated under these conditions, we extracted the surface layer of untreated and bile-treated parasites by incubation in CTAB. A number of membrane bound proteins were phosphorylated in untreated larvae (Fig. 5, lane 1), and this was diminished by genistein (lane 2) and enhanced by sodium orthovanadate (lane 3). The data therefore suggest that tyrosine phosphorylation of surface proteins is also regulated by ecto-enzymes. Enhanced phosphorylation of certain proteins by okadaic acid (lane 6) suggested that serine/threonine phosphorylation was also occurring on surface bound proteins, although staurosporine and A-3 had a negligible effect (lanes 4 and 5). Following bile treatment, a different pattern of phosphorylation was evident, with the major phosphoprotein resolving at about 45 kDa (lanes 7–12). This protein was not observed in untreated larvae (lanes 1–6) and is therefore only available for phosphorylation following parasite activation. The use of inhibitors clearly demonstrated that this was effected by a serine/threonine kinase (lanes 10 and 11), and that this is regulated by a corresponding phosphatase (lane 12). The surface specificity of this process was further verified by the complete lack of phosphoproteins in the remaining parasite carcasses (data not shown).

We have previously shown that the surface 'accessory layer' of *T. spiralis* infective larvae is composed of at least two layers of lipids in an unusual inverted hexagonal type II configuration (Hex-II) [5]. We therefore carried out an ultrastructural examination by freeze fracture and thin section electron microscopy following exposure to bile in order to investigate whether this induced ultrastructural changes, and whether the altered profiles of protein phosphorylation could be accounted for by the exposure of a new surface.

Examination of the parasite by thin section electron microscopy highlighted no differences in appearance before or after bile treatment, and the accessory layer was present in all samples (Fig. 6B,D). However, freeze fracture analysis revealed that the organisation of surface lipids was altered by bile treatment in that the Hex-II configuration (Fig. 6A) was no longer present, and the morphology observed resembled that of a normal bilayer with incorporated proteins (Fig. 6C). Therefore, under the conditions used in these experiments, incubation of infective larvae in bile does not lead to the exposure of a new surface, although the conformation of the surface membrane is reorganised.

4. Discussion

We have demonstrated that infective larvae of the intracellular parasitic nematode *T. spiralis* possess an active reversible protein phosphorylation system on their surface. Serine/threonine protein kinases, tyrosine kinases, and respective protein phosphatases all appear to be present, and can act on secreted and membrane bound parasite substrates (Figs. 1 and 5). The use of inhibitors demonstrated that the enzymes involved have significantly different substrate specificities, but the identity of the phosphorylated proteins is unknown. Indeed, although *T. spiralis* infective larvae are known to secrete a complex set of proteins, with the exception of protein kinase [3] and nuclease [6,7] activities, little is known of their functional properties.

It was important to establish that the protein phosphorylation observed was indeed due to the action of ecto-enzymes

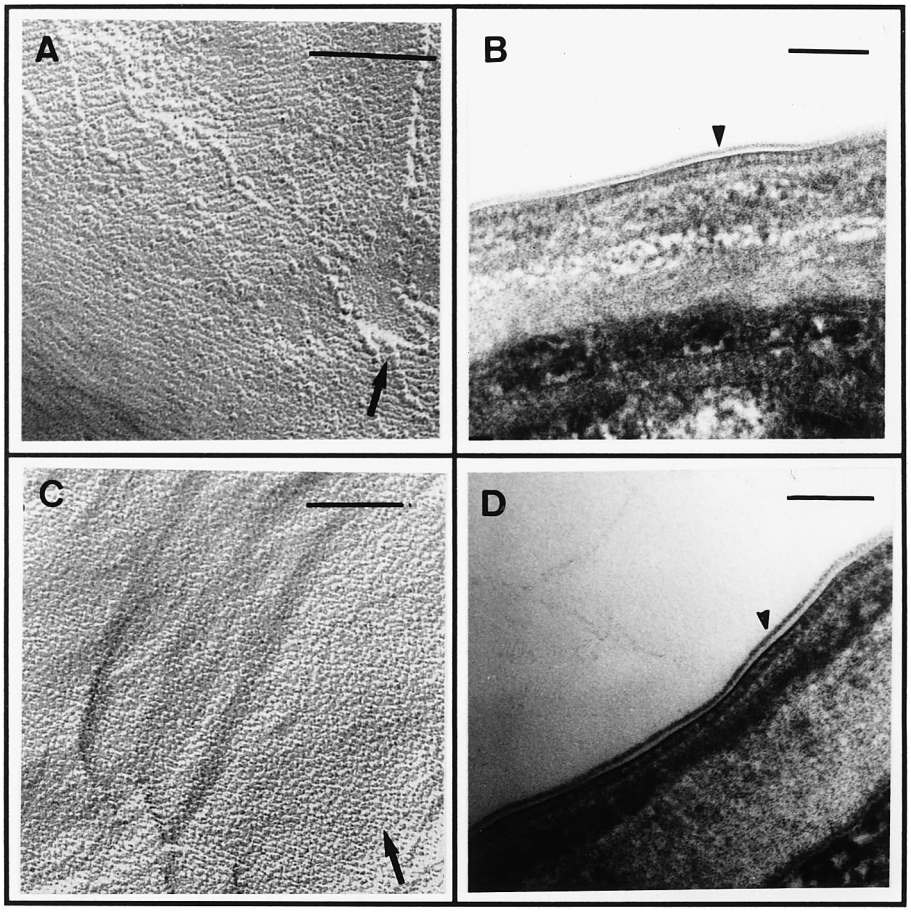

Fig. 6. Freeze fracture (A and C) and thin section (B and D) electron micrographs of replicas obtained from untreated (A and B) and bile-treated (C and D) parasites. The bar represents 150 nm. Arrowheads indicate the accessory layer, and arrows the direction of shadowing.

and not due to (a) interaction of [γ-^{32}P]ATP with extrinsic proteins via ATP-binding sites, (b) the hydrolysis of [γ-^{32}P]ATP by ecto-ATPases and uptake of radiolabelled inorganic phosphate ($^{32}P_i$) by the parasite or (c) the direct uptake of [γ-^{32}P]ATP by the parasite, both leading to the phosphorylation and subsequent release of intracellular proteins. The lack of phosphorylation in the presence of [α-^{32}P]ATP effectively discounts the first possibility. Protein phosphorylation was also not observed with $^{32}P_i$, excluding the possibility of intracellular phosphorylation events and release of labelled proteins to the exterior. Furthermore, phosphorylation was inhibited by exogenously added ATP-consuming enzymes, which would not interfere with intracellular events. In addition, we observed no labelling of somatic parasite proteins after the surface membranes had been removed with CTAB. Thus, we conclude that the protein phosphorylation observed is indeed due to the action of ecto-enzymes.

The altered profile of protein phosphorylation induced by exposure of parasites to bile could be interpreted as resulting from modification of existing activities, activation of novel enzymes or access to different substrates. This was particularly pronounced when considering membrane bound phosphoproteins, as a major species at 45 kDa, clearly responsive to serine/threonine phosphorylation, was observed only after bile treatment. The identity of this protein is not known, but it is reasonable to hypothesise that it may be involved in some recognition or activation process in the enteral phase of infection. We have recently shown that *T. spiralis* larvae secrete at least two distinct serine/threonine protein kinases, which almost exclusively phosphorylate a protein doublet at 50–55 kDa [3]. This is a distinct event from that effected by the ecto-enzymes reported here, as phosphorylation of the 50–55 kDa doublet could not efficiently be blocked by the use of the protein kinase inhibitors utilised in the current study, and furthermore we found no evidence for the presence of secreted protein phosphatases [3].

It has previously been reported that prolonged exposure to bile and trypsin induces shedding of the accessory layer of *T. spiralis* larvae, and induces a change in their motility from a coiling/uncoiling pattern to sinusoidal movement [8]. We also observed this change in motility when parasites were treated solely with bile, but the 'accessory layer' was not shed. We did however observe a complete structural reorganisation of the surface, as the non-bilayer Hex-II configuration was lost and replaced by a more traditional bilayer-like appearance. We therefore conclude that bile treatment which induces infectivity modulates the activity of the enzymes involved in the phosphorylation process and results in the exposure of new substrates.

Ecto-protein kinase and phosphatase activities have been identified on a variety of cells [9,10] and a number of different pathogenic organisms such as the intracellular parasitic protozoans *Leishmania* and *Trypanosoma* spp. The physiological roles for these protozoal enzymes are undefined, although it

has been suggested that they may be involved in invasion, cell cycle regulation or the inhibition of various cellular processes such as the respiratory burst [11,12]. To our knowledge, this is the first demonstration of such ecto-enzymes on helminth parasites. An integral membrane protein with serine/threonine kinase activity has been detected on the surface of the trematode parasite *Schistosoma mansoni*, although the catalytic domain is predicted to be cytoplasmic. As the protein is homologous to the TGF_b receptor family, this protein presumably acts to transduce signals by an unidentified ligand(s) [13].

Activation of signal transduction pathways necessary for invasion of pathogens is invariably associated with protein phosphorylation of cellular targets [4], and tyrosine kinase inhibitors block invasin-mediated uptake of several bacterial species [14]. In the cases of *Trypanosoma cruzi* and *Theileria parva*, protein phosphorylation events in both the parasite and the host cell are essential for invasion [15,16]. Bacterial pathogens also secrete protein kinases, and one such serine/threonine-directed enzyme is encoded on a virulence plasmid in *Yersinia* spp. [17], the products of which are thought to form a multi-protein 'attack complex' on the bacterial surface.

The mechanism of cellular invasion by *T. spiralis* is unknown, and the role of secreted or surface bound proteins with respect to invasion and survival of the parasite in either skeletal muscle or intestinal epithelia remains largely uninvestigated. Cells from five mammalian species have been shown to be susceptible to invasion by infective larvae, reflecting the broad host range of the parasite. Intestinal and kidney epithelial cell lines were penetrated, although fibroblasts and myoblasts were resistant to invasion [1,18]. This selectivity suggests the involvement of an as yet undefined interaction between parasites and cells. This may depend upon the capacity for specific signal transduction processes in epithelial cells and/or direct interactions between parasite secreted or surface bound ecto-enzymes and host cell molecules. We are now working to determine whether the enzyme systems identified in this communication play a role in invasion of host epithelial cells.

Acknowledgements: This work was supported by the Wellcome Trust and the BBSRC. V.P.S. was funded by a Wellcome Trust Prize Fellowship (048638/Z/96/Z).

References

[1] Despommier, D.D. (1983) in: *Trichinella* and Trichinosis (Campbell, W.C., Ed.), pp. 75–151, Plenum Press, New York.
[2] ManWarren, T., Gagliardo, L., Geyer, J., McVay, C., Pearce-Kelling, S. and Appleton, J. (1997) Infect. Immun. 65, 4806–4812.
[3] Arden, S.R., Smith, A.M., Booth, M.J., Tweedie, S., Gounaris, K. and Selkirk, M.E. (1997) Mol. Biochem. Parasitol. 90, 111–119.
[4] Bliska, J.B., Galan, J.E. and Falkow, S. (1993) Cell 73, 903–920.
[5] Gounaris, K., Smith, V.P. and Selkirk, M.E. (1996) Biochim. Biophys. Acta 1281, 91–100.
[6] Mak, C.H. and Ko, R.C. (1999) Eur. J. Biochem. 260, 477–481.
[7] Mak, C.H., Chung, Y.Y.Y. and Ko, R.C. (2000) Parasitology 120, 527–533.
[8] Steward, G.L., Despommier, D.D., Burnham, J. and Raines, K.M. (1987) Exp. Parasitol. 63, 195–204.
[9] Walter, J., Schnolzer, M., Pyerin, W., Kinzel, V. and Kubler, D. (1996) J. Biol. Chem. 271, 111–119.
[10] Petitfrere, E., Sartelet, H., Aguie-Aguie, G., Martiny, L. and Haye, B. (1996) Biochem. Biophys. Res. Commun. 220, 746–753.
[11] Sacerdoti-Sierra, N. and Jaffe, C.L. (1997) J. Biol. Chem. 272, 30760–30765.
[12] Furuya, T., Zhong, L., Meyer-Fernandes, J.R., Lu, H.-G., Moreno, S.N.J. and Docampo, R. (1998) Mol. Biochem. Parasitol. 92, 339–348.
[13] Davies, S.J. and Pearce, E.J. (1995) Mol. Biochem. Parasitol. 70, 33–44.
[14] Rosenshine, I., Duronio, V. and Finlay, B.B. (1992) Infect. Immun. 60, 2211–2217.
[15] Vieira, M.C., de Carvalho, T.U. and de Souza, W. (1994) Biochem. Biophys. Res. Commun. 203, 967–971.
[16] Michael, K. and Shaw, M.K. (1996) Exp. Parasitol. 84, 344–354.
[17] Galyov, E.E., Hakansson, S., Forsberg, A. and Wolf-Watz, H. (1993) Nature 361, 730–732.
[18] Butcher, B.A., Gagliardo, L.F., ManWarren, T. and Appleton, J.A. (2000) Mol. Biochem. Parasitol. 107, 207–218.